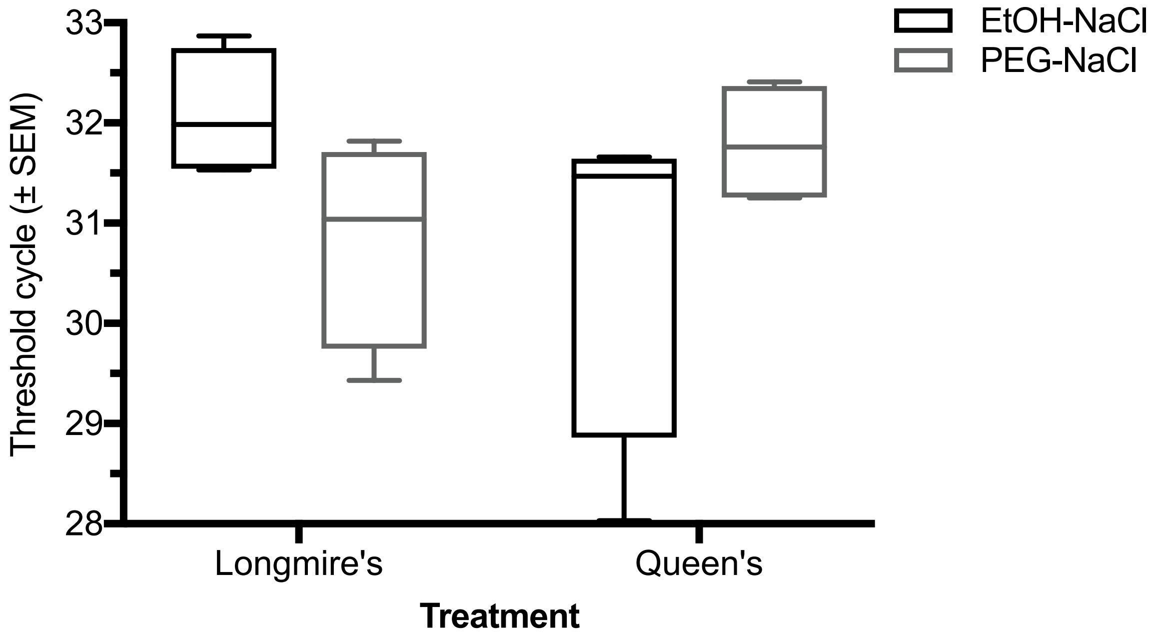
Supplemental Figure S1

Methods

30 mL water samples were collected in 50 mL LoBind® tubes from TropWATER tilapia tank (see Sources of genomic, synthetic, and environmental DNA) and immediately preserved with 10 mL Longmire's or 10 mL Queen's (Table 1). Longmire's and Queen's preserved tank water samples (n = 8 each) were cooled to room temperature after 9 days at constant 40˚C, pelleted in swinging-bucket rotor (3,270 x g, 60 min, 4˚C; Allegra X12R centrifuge with SX4750 rotor, Beckman Coulter Pty Ltd., Australia), resuspended in 600 µL Lysis Buffer I (Table 3), frozen at ≤ -20˚C for ≥ 30min, thawed at room temperature for ≥ 30 min, intensely vortexed at 2,600 rpm for 30 sec (beat-beating alternative; Lever, et al., 2015), and lysed at 50˚C for 1 hr. Samples were then purified once with PCI and twice with CI by vortex emulsification (10 sec), phase separation (10 min, 10,000 x g, 4°C), and supernatant retention. Samples were then subjected to terminal precipitation overnight (4˚C) with 55.5 µg/mL glycogen and either 2 volumes EtOH with 270 mM NaCl (n = 4 Longmire's, n = 4 Queen's) or 2 volumes PEG8000-NaCl (Table 2; n = 4 Longmire's, n = 4 Queen's). Samples were then pelleted in fixed angle microcentrifuge (20,000 x g, 30 min, 20˚C), washed twice with 70% EtOH (vortex followed by 10,000 x g for 10 min at 4°C), eluted in 100 µL water, and assessed for *O. mossambicus* eDNA without inhibitor purification using initial tilapia assay (see Supplemental Information).

Results

Detection of *O. mossambicus* eDNA was 100% across all treatments. Two-way ANOVA revealed no significant effect of EtOH-NaCl versus PEG8000-NaCl precipitation ($F_{1,12}$ = 0.01206, P = 0.9144), Longmire's versus Queen's preservation ($F_{1,12}$ = 0.1794, P = 0.6794), or their interactions ($F_{1,12}$ = 4.62, P = 0.0527).



Supplemental Figure S1. Comparison of threshold cycle (C_t) values obtained using initial tilapia assay (see Section 2.4) on 15 mL unfiltered water samples collected from *O. mossambicus* tank (see Sources of genomic, synthetic, and environmental DNA) that were concurrently preserved using 5 mL Longmire's or 5 mL Queen's (Table 1) and subjected to discrete terminal precipitation using ethanol and sodium chloride (EtOH-NaCl) or polyethylene glycol 8000 and sodium chloride (PEG-NaCl; Table 2). Two-way ANOVA revealed no significant effect of EtOH-NaCl versus PEG-NaCl precipitation ($P > 0.9$), Longmire's versus Queen's preservation ($P > 0.6$), or their interactions ($P > 0.05$). Line in box represented median value while box and whiskers represent the four quartiles.

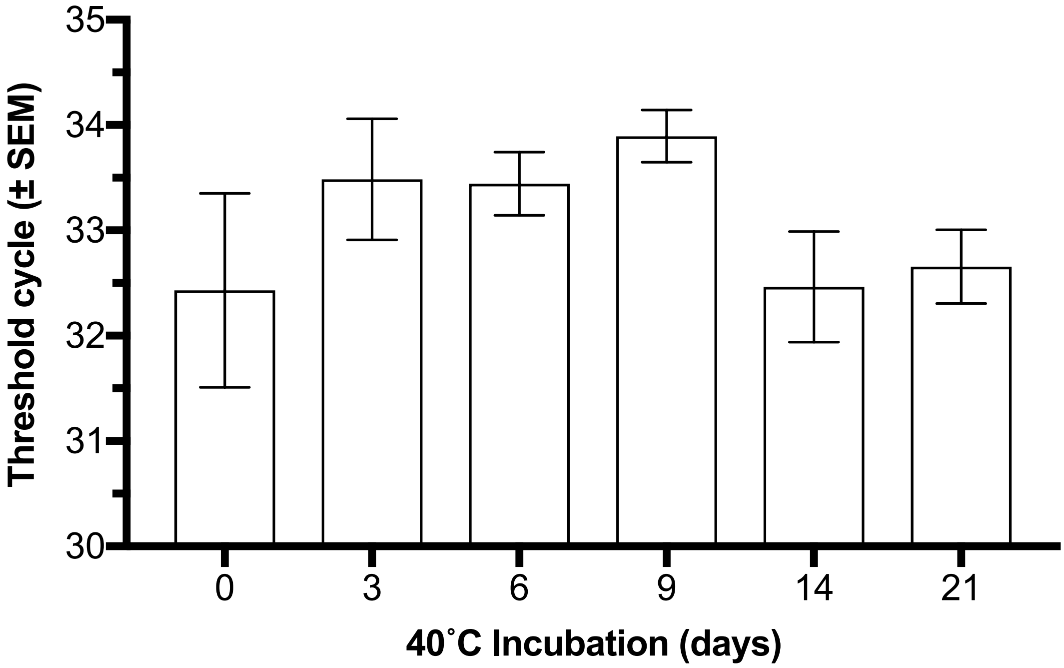
Supplemental Figure S2

Methods

15 mL unfiltered water samples were collected in 50 mL LoBind® tubes from TropWATER tilapia tank (see Sources of genomic, synthetic, and environmental DNA) and preserved by mixing with 5 mL Longmire's Solution (1:3 ratio) before 40˚C incubation for 0, 3, 6, 9, 14, or 21 days (*n* = 3 per time-point). At each time-point, 20 mL preserved tank water samples were initially precipitated overnight at 4˚C with 0.8 volumes absolute isopropanol and 0.2 volumes 5M NaCl, pelleted in swinging-bucket rotor at 3,270 x g for 90 min at 22˚C, resuspended in 600 µL Lysis Buffer I (Table 3), frozen at ≤ -20˚C for ≥ 30min, thawed at room temp for ≥ 30 min, intensely vortexed at 2600 rpm for 30 secs (beat-beating alternative), lysed at 50˚C for 1 hour, PCI purified, incubated overnight at 4˚C in 2 volumes PEG8000-NaCl (Table 2), pelleted in fixed angle microcentrifuge (20,000 x g, 30 min, 20˚C), washed twice with 70% EtOH, eluted in 100 µL water, and assessed for *O. mossambicus* eDNA presence without inhibitor purification using initial tilapia assay (see Supplemental Information).

Results

Detection of *O. mossambicus* eDNA was 100% at Days 0, 3, 6, 9, 14, and 21. One-way ANOVA revealed that *O. mossambicus* eDNA abundance did not change across 21 days at 40˚C ($F_{5,16} = 1.799$, $P = 0.17$).



Supplemental Figure S2. Comparison of threshold cycle (C_t) values obtained when 15 mL unfiltered water samples collected from *O. mossambicus* tank (see Sources of genomic, synthetic, and environmental DNA) were preserved with 5 mL Longmire's (see Table 1) and subjected to 40˚C incubation for 21 days and assessed with initial tilapia assay (see Supplemental Information). One-way ANOVA revealed a non-significant difference in *O. mossambicus* eDNA abundance across time ($P > 0.15$). Bars represent mean ± SEM.

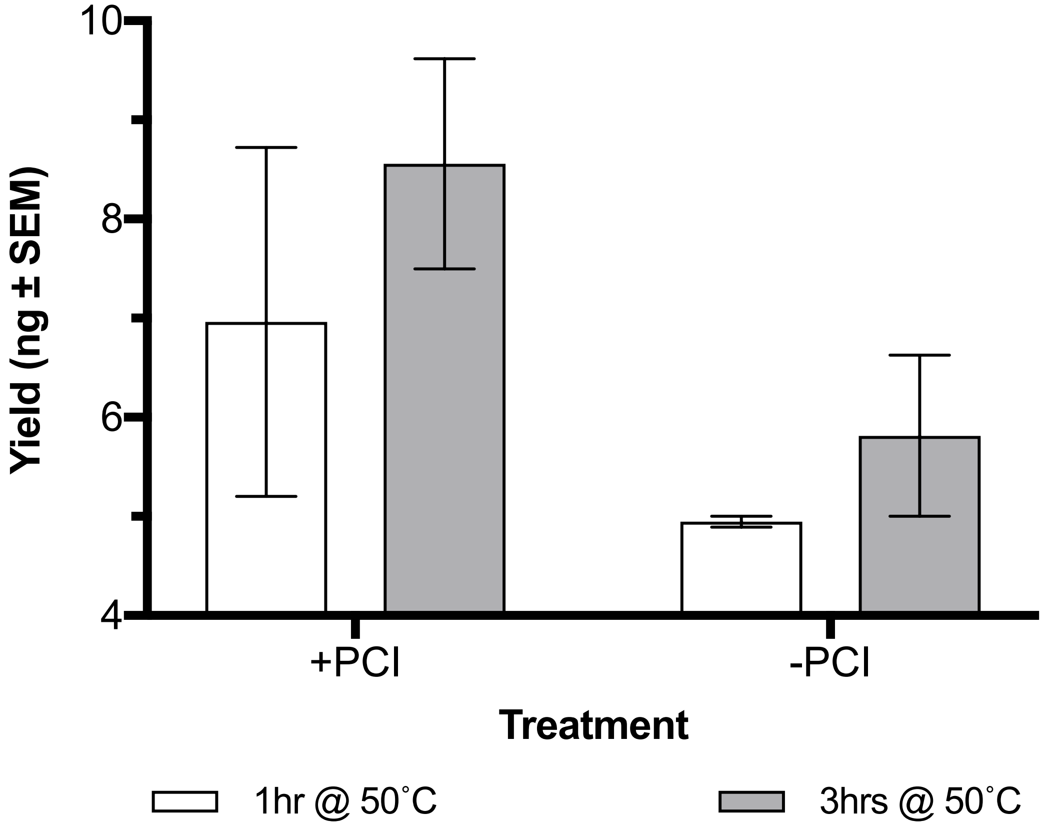
Supplemental Figure S3

Methods

15 mL water samples were collected in 50 mL LoBind® tubes from TropWATER tilapia tank (see Sources of genomic, synthetic, and environmental DNA) and preserved by mixing with 5 mL Longmire's (1:3 ratio) before 40˚C incubation for 1 hour (n = 4). 20 mL preserved tank water samples were initially precipitated overnight at 4˚C with 0.8 volumes absolute isopropanol, 0.2 volumes 5M NaCl, and 4.4 µg/mL commercial glycogen (Sigma-Aldrich Pty Ltd, Australia), pelleted in swinging-bucket rotor (3,270 x g, 90 min, 22˚C; Allegra X12R centrifuge with SX4750 rotor, Beckman Coulter Inc., Australia), resuspended in 600 µL Lysis Buffer I (Table 3), frozen at ≤ -20˚C for ≥ 30min, thawed at room temperature for ≥ 30 min, intensely vortexed at 2600 rpm for 30 sec (beat-beating alternative), lysed at 50˚C for 1 hour (n = 2) or 3 hours (n = 2), and subjected to one PCI and two CI purifications (+PCI; n = 2; see Section 2.7.3) or not (-PCI; n = 2) before overnight precipitation at 4˚C with 2 volumes PEG8000-NaCl and 55.5 µg/mL commercial glycogen. Samples were pelleted in fixed angle microcentrifuge (20,000 x g, 30 min, 20˚C), washed twice with 70% EtOH, eluted in 100 µL water, and assessed for *O. mossambicus* eDNA presence without inhibitor purification using initial tilapia assay (see Supplemental Information). Yields were extrapolated using the initial tilapia assay gDNA standard curve that was included on the same plate (E = 96.0%, R^2 = 0.983).

Results

Detection of *O. mossambicus* eDNA was 100% for all treatments. Two-way ANOVA revealed no significant effect of lysis duration ($F_{1,4}$ = 1.248; P = 0.3265), PCI purification ($F_{1,4}$ = 4.635; P = 0.0977), or their interaction ($F_{1,4}$ = 0.109; P = 0.7578).



Supplemental Figure S3. Comparison of extrapolated *O. mossambicus* eDNA yield obtained when 15 mL unfiltered water samples collected from *O. mossambicus* tank (see Sources of genomic, synthetic, and environmental DNA) was preserved with 5 mL Longmire's (see Table 1) and subjected to 50˚C lysis for 1 hour or 3 hours prior to additional purification with phenol:chloroform:isoamyl alcohol (+PCI) or without (-PCI; see Section 2.7.5). Samples were assessed with initial tilapia assay (see Supplemental Information). Two-way ANOVA revealed that neither 1 hour or 3 hours lysis nor +PCI or -PCI purifications had a significant effect on *O. mossambicus* eDNA yield ($P > 0.09$). Bars represent mean ± SEM.

Supplemental Figure S4

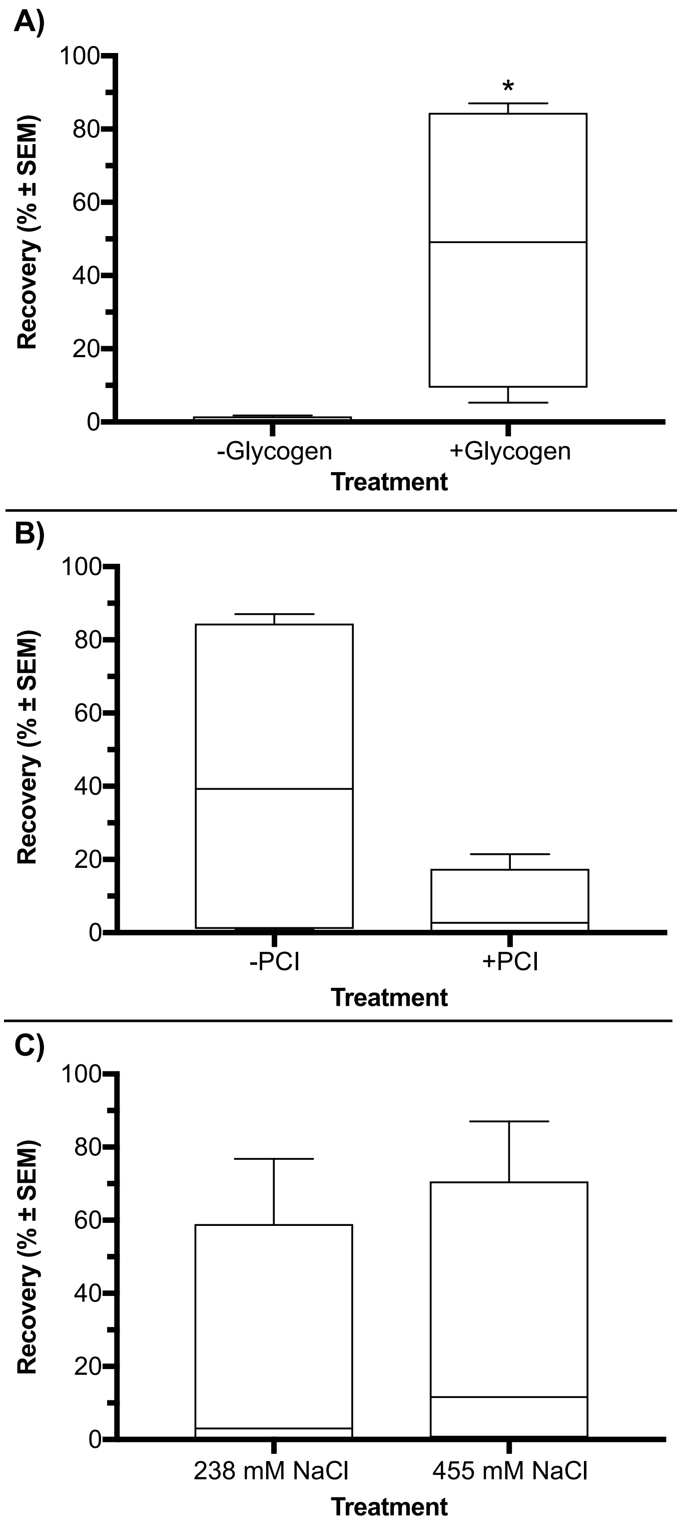
Methods

15 mL DNA-free water samples (MilliQ; Thermo Fisher Scientific Australia Pty Ltd, Scoresby VIC, Australia) were buffered with 5 mL Longmire's (1:3 ratio) in 50 mL LoBind® tubes and then spiked with 25 ng *O. mossambicus* gDNA (*n* = 8) before incubation at 40˚C for 3 hours ("high-copy clean-spike"). Samples were initially precipitated overnight at 4˚C with 0.8 volumes absolute isopropanol, either 0.1 volume 5M NaCl (238 mM final, *n* = 4; Doi, et al., 2017) or 0.2 volumes 5M NaCl (455 nM final, *n* = 4; Table 2), and either 4.4 µg/mL commercial glycogen (+glycogen, *n* = 4) or no glycogen (-glycogen, *n* = 4). Samples were then pelleted in swinging-bucket rotor (Allegra X12R centrifuge with SX4750 rotor; Beckman Coulter Australia) at 3,270 x g for 90 min at 22˚C, resuspended in 600 µL Lysis Buffer I (Table 3), frozen at ≤ -20˚C for ≥ 30 min, thawed at room temperature for ≥ 30 min, intensely vortexed at 2600 rpm for 30 sec (beat-beating alternative), lysed at 50˚C for ≥ 3hrs, and subjected to one PCI and two CI purifications (+PCI; *n* = 4; see Section 2.7.3) or not (-PCI; *n* = 4) before overnight precipitation at 4˚C with 2 volumes PEG8000-NaCl and 55.5 µg/mL commercial glycogen (+glycogen, *n* = 4) or without glycogen (-glycogen; *n* = 4). Samples were pelleted in a fixed angle microcentrifuge (20,000 x g, 30 min, 20˚C), washed twice with 70% EtOH, eluted in 100 µL water, and assessed for *O. mossambicus* gDNA recovery without inhibitor purification using initial tilapia assay (see Supplemental Information). Yields were extrapolated using the initial tilapia assay gDNA standard curve that was included on the same plate (E = 101.7%, R^2 = 0.998).

Results

Detection of *O. mossambicus* eDNA was 100% for all high-copy tank-spike treatments. Two-tailed Mann-Whitney U tests (Bonferroni-corrected alpha = 0.0288) demonstrated that the inclusion versus exclusion of glycogen yielded significantly higher *O. mossambicus* gDNA recovery (P = 0.0286; Supplemental Figure 4A) whereas inclusion versus exclusion of PCI purification (+PCI versus -PCI) did not have a significant effect on *O. mossambicus* gDNA recovery (P = 0.3429; Supplemental Figure 4B) nor did addition of 0.1 volume or 0.2 volumes 5M NaCl (238 mM or 455 mM final) to initial isopropanol precipitation (P = 0.6857; Supplemental Figure 4C), respectively. Minimum to maximum *O. mossambicus* gDNA recovery efficiency varied depending on +glycogen versus -glycogen (5.3 – 87.0% versus 0.05

– 1.78%), +PCI versus -PCI (0.05 – 21.46% versus 0.75 – 87.0%), and 0.1 volume versus 0.2 volumes 5M NaCl (0.05 – 76.8% versus 0.17 – 87.0%), respectively. The overall highest *O. mossambicus* gDNA recovery of 87.0% was obtained when: 1) 4.4 µg/mL and 55.5 µg/mL glycogen were included in initial and terminal precipitations, respectively, 2) 0.2 volumes 5M NaCl was added to initial precipitation, and 3) PCI purification following lysis was excluded.



Supplemental Figure S4. Comparison of *O. mossambicus* gDNA spike-in recovery obtained when the following PPLPP workflow variations were empirically tested: A) glycogen exclusion (-glycogen) versus glycogen inclusion (+glycogen; 4.4 µg/mL and 55.5 µg/mL in initial and terminal precipitations, respectively), B) phenol:chloroform:isoamyl alcohol (PCI) purification exclusion (-PCI) versus inclusion (+PCI), and C) addition of 0.1 volume 5M NaCl (238 mM NaCl final) or 0.2 volumes 5M NaCl (455 mM NaCl final) during initial isopropanol precipitation. Astrict (panel A) denotes a significant pairwise difference (two-tailed Mann-Whitney U test $P < 0.03$). Line in box represented median value while box and whiskers represent the four quartiles.

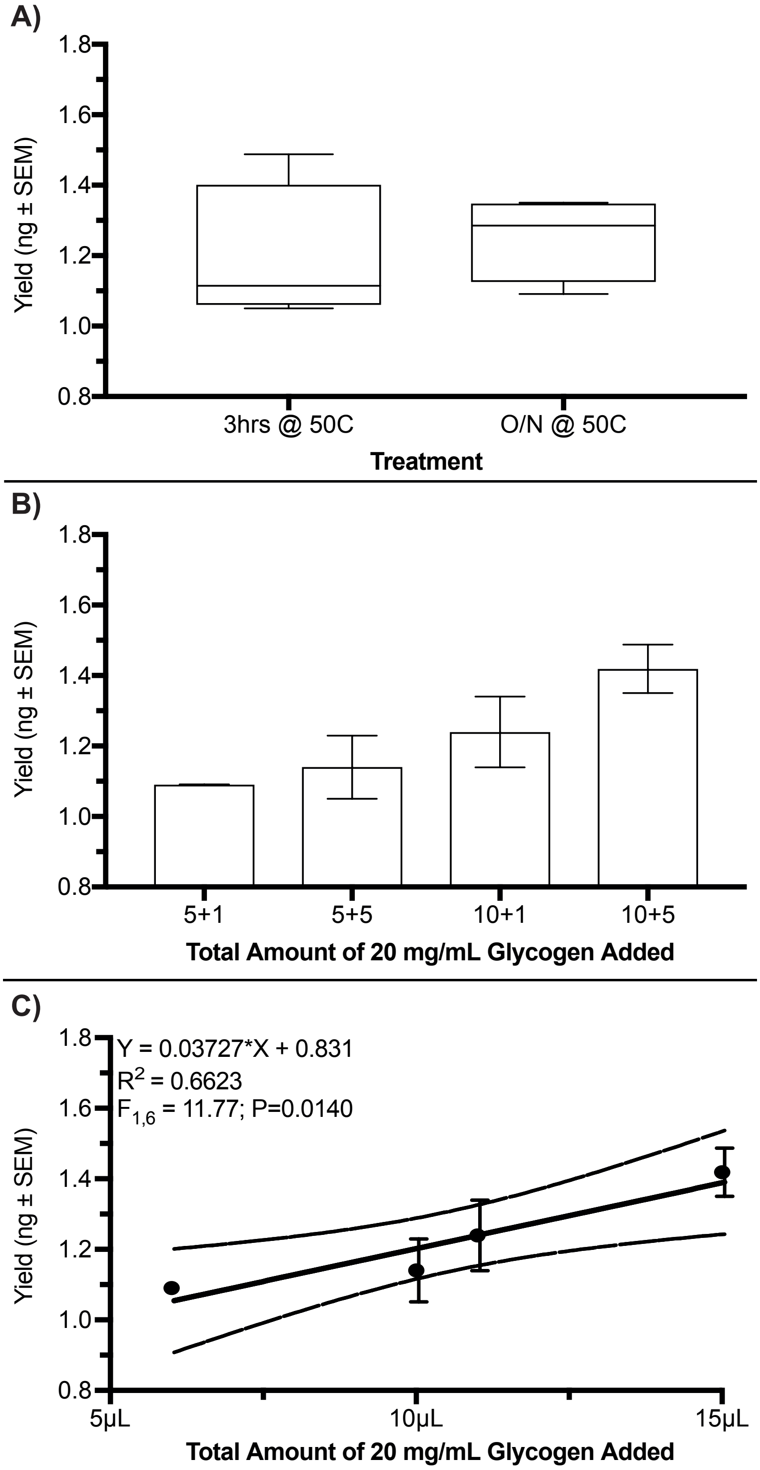
Supplemental Figure S5

Methods

15 mL water samples were collected in 50 mL LoBind® tubes from TropWATER tilapia tank (see Sources of genomic, synthetic, and environmental DNA) and preserved by mixing with 5 mL Longmire's (1:3 ratio) before immediate extraction (n = 8). Samples were initially precipitated overnight at 4˚C with 0.8 volumes absolute isopropanol, 0.2 volumes 5M NaCl, and either 2.2 µg/mL glycogen (5 µL of 20 mg/mL commercial glycogen, n = 4) or 4.4 µg/mL glycogen (10 µL of 20 mg/mL commercial glycogen, n = 4). Samples were then pelleted in swinging-bucket rotor (Allegra X12R centrifuge with SX4750 rotor; Beckman Coulter Australia) at 3,270 x g for 90min (22˚C), resuspended in 600 µL Lysis Buffer I (Table 3), frozen at ≤ -20˚C for ≥ 30min, thawed at room temperature for ≥ 30 min, intensely vortexed at 2600 rpm for 30 sec (beat-beating alternative), and lysed at 50˚ for either 3 hours (n = 4) or overnight (n = 4) before overnight precipitation at 4˚C with 2 volumes PEG8000-NaCl and 11.1 µg/mL glycogen (1 µL of 20 mg/mL commercial glycogen, n = 4) or 55.5 µg/mL glycogen (5 µL of 20 mg/mL commercial glycogen, n = 4). Samples were pelleted in fixed angle microcentrifuge (20,000 x g, 30 min, 20˚C), washed twice with 70% EtOH, eluted in 100 µL water, and assessed for *O. mossambicus* gDNA recovery without inhibitor purification using initial tilapia assay (see Supplemental Information). Yields were extrapolated using the initial tilapia assay gDNA standard curve that was included on the same plate (E = 92.49%, R^2 = 0.996).

Results

Detection of *O. mossambicus* eDNA was 100% for all treatments. Two-tailed Welch's t test confirmed that there is no increase or decrease in eDNA yield obtained following 3 hours versus overnight lysis at 50˚C (t = 0.5186, df = 4.92; P = 0.6265; Supplemental Figure 5A). Accordingly, lysis treatments were combined for subsequent one-way ANOVA and linear regression analyses on *O. mossambicus* eDNA yield (ng ± SEM) obtained for each cumulative glycogen amount added to initial plus terminal precipitations (5µL+1µL, 5µL+5µL, 10µL+1µL, and 10µL+5µL), respectively. One-way ANOVA revealed a non-significant effect of glycogen amount on eDNA yield ($F_{3, 4}$ = 3.683; P = 0.1201; Supplemental Figure 5B); however, linear regression revealed a significant correlated between eDNA yield and total amount of glycogen added during extraction ($F_{1,6}$ = 11.77; P = 0.0140; R^2 = 0.6623; Supplemental Figure 5C). Therefore, glycogen at ≥ 4.4 µg/mL and ≥ 55.5 µg/mL in initial and terminal precipitations, respectively, were included in all subsequent PPLPP workflow extractions (Figures 3 – 5; Supplemental Figures 6 – 8).



Supplemental Figure S5. Comparison of *O. mossambicus* eDNA yield obtained when 15 mL unfiltered water samples collected from *O. mossambicus* tank (see Sources of genomic, synthetic, and environmental DNA) were preserved with 5 mL Longmire's and subjected to 50˚C lysis incubation for 3 hours versus overnight (O/N; panel A). Given the non-significant difference ($P > 0.6$) lysis time treatments were combined and assessed by one-way ANOVA (B) and linear regression (C) to determine the effect of cumulative 20 mg/mL glycogen volume added to initial plus terminal precipitations (5µL+1µL, 5µL+5µL, 10µL+1µL, and 10µL+5µL), respectively. One-way ANOVA was non-significant ($P > 0.1$) whereas linear regression was significant. Line in box represented median value while box and whiskers represent the four quartiles (A) while bars and dots (B and C) represent mean ± SEM.

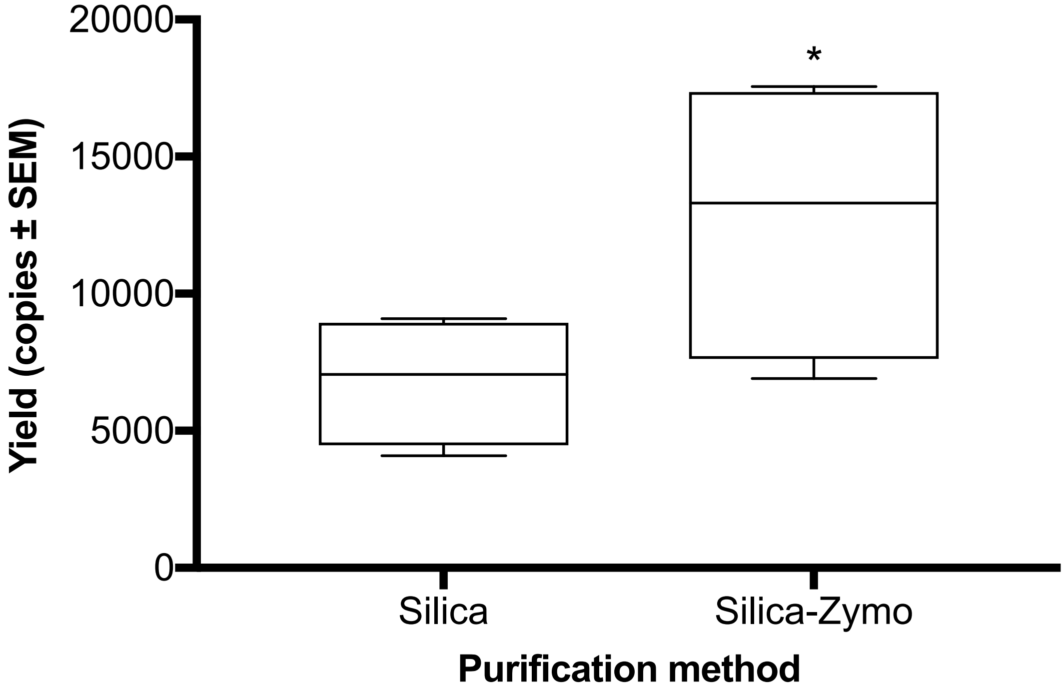
Supplemental Figure S6

Methods

The effectiveness of silica only versus silica-Zymo double inhibitor purification of *O. mossambicus* eDNA was then assessed using 15 mL unfiltered water samples (*n* = 4) collected *O. mossambicus* tank in 50 mL LoBind® tubes (see Sources of genomic, synthetic, and environmental DNA). Samples were extracted following PPLPP workflow (without optional PCI purification; Figure 2) using 4.4 µg/mL and 111.1 µg/mL in-house glycogen in initial and terminal precipitations, respectively. Initial isopropanol precipitation was conducted at 12,104 x g for 5 min at 20˚C in fixed-angle rotor (Heraeus Megafuge 8R with HighConicIII rotor; Thermo Fisher Scientific Australia Pty Ltd, Scoresby VIC, Australia). Samples were eluted in 200 µL water. 100 µL was inhibitor purified using silica (Supplemental Protocol 3) and then the remaining volume of each silica purified sample (≈ 88 µL) was subsequently purified using Zymo (silica-Zymo). Recovered *O. mossambicus* eDNA quantity was determined using Tilapia_v2_16S with subsequent extrapolation from *O. mossambicus* sDNA standard curve (Table 4). All *O. mossambicus* tank water samples that produced amplicons with ΔT_m inside 99.7% confidence interval of *O. mossambicus* gDNA standards (Table 4) were considered positive detections without Sanger sequencing confirmation given the sole presence of *O. mossambicus* within sampled tank water.

Results

Detection of *O. mossambicus* eDNA was 100% for both treatments (Supplemental Figure 6). Moreover, double inhibitor purification (silica-Zymo) yielded significantly more *O. mossambicus 16S* copies than inhibitor purification with silica only (2-tailed Welch's t test: t = 4.055, DF = 3, P = 0.0270).



Supplemental Figure S6. *Oreochromis mossambicus* eDNA yield recovered from unfiltered water samples collected from TropWATER facility *O. mossambicus* tank (see Sources of genomic, synthetic, and environmental DNA) that were assessed by qPCR following silica purification only ("Silica"; Supplemental Protocol 2) or silica purification with subsequent OneStep™ PCR Inhibitor Removal Column purification ("Silica-Zymo"; Zymo Research Inc., California USA). Yield was determined using Tilapia_v2_16S under optimal conditions (Table 4). Astrict (*) denotes a significant increase in *O. mossambicus 16S* copies recovered following Silica-Zymo double purification compared to Silica only purification (2-tailed Welch's t test P = 0.0270). Moreover, the 100% detection rate for *O. mossambicus* despite ≈ 50% lower yield following Silica only purification (i.e., ≈ 1 qPCR cycle) demonstrates that target eDNA is not lost during silica purification but rather that this form of inhibitor purification is subject to potential silica carry-over, which can inhibit enzymatic reactions. Accordingly, subsequent Zymo (or other) purification is recommended to ensure removal of any carried-over silica. Line in box represented median value while box and whiskers represent the four quartiles.

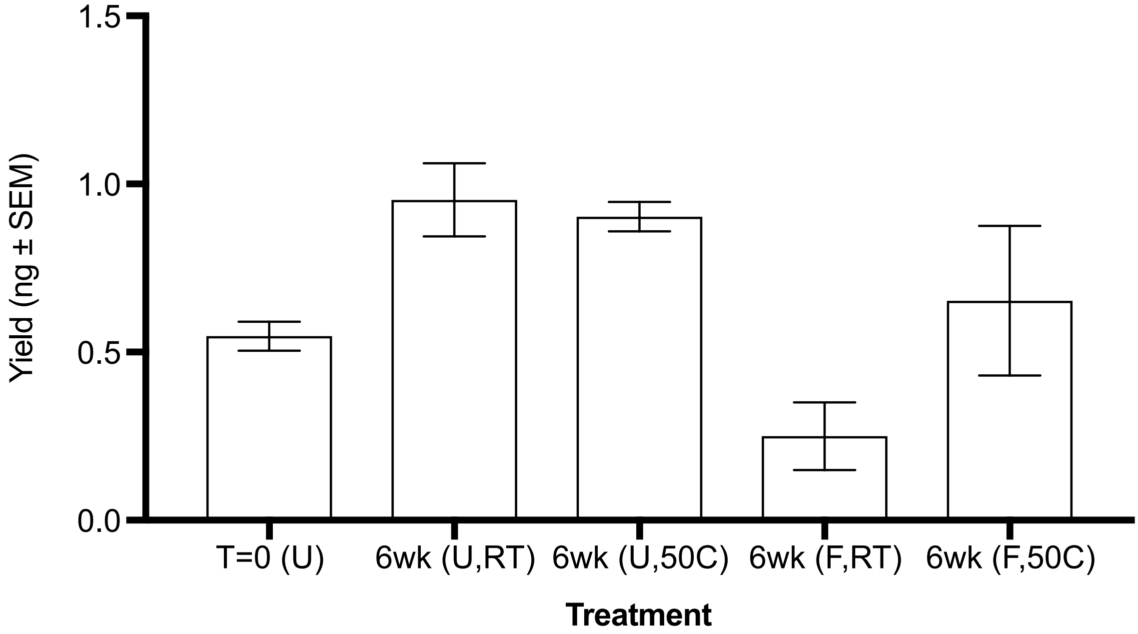
Supplemental Figure S7

Methods

Unfiltered samples were collected from *O. mossambicus* tank (see Sources of genomic, synthetic, and environmental DNA) on 22 March 2018 by decanting 15 mL from discrete water grabs into 50 mL LoBind® tubes (n = 10) preloaded with 5 mL Longmire's (Table 1) followed by five inversions to ensure complete mixture. Filtered samples were collected from *O. mossambicus* tank (n = 6) by passing discrete 15 mL water grabs through pre-sterilized 1.2 µM nylon net membrane filters (Merck Millipore Ltd Pty, Australia) using Grover® portable peristaltic pump (Grover Scientific Pty Ltd, Australia). Each filter was immediately rolled and cut in half with ethanol-sterilized scissors and each filter half (n = 12) was preserved by submersion in Longmire's that was pre-loaded into new 2 mL LoBind® tubes. Samples were transported back to MEEL at ambient temperature (≈ 24˚C). In light of the unknown cell-type composition of eDNA shed by *O. mossambicus* (i.e., ratio of cell-free to cell-bound) and the potential loss of cell-free eDNA when membrane filters ≥ 0.2 µM are used (Turner, Barnes, Xu, Jones, Jerde, and Lodge. "Particle size distribution and optimal capture of aqueous macrobial eDNA". *Methods in Ecology and Evolution*. 2014; 5(7): 676-684), baseline *O. mossambicus* eDNA yield (T = 0) was determined using unfiltered water samples (n = 4) extracted within three days of collection. Remaining unfiltered and filter half samples were incubated upright without agitation on benchtop at room temperature (n = 3 and n = 6) and in a dry oven (BFD 53; Binder GmbH, Germany) set to 50˚C (n = 3 and n = 6), respectively. 50 mL LoBind® tubes used to collected unfiltered water samples were submerged to base of lid in 10% bleach to decontaminate prior to incubation commencement. Unfiltered samples were incubated in open top racks whereas filtered samples were incubated in closed plastic freezer boxes. Unfiltered samples were submerged to base of lid in 10% bleach again after 39 days at 50˚C to remove any potential DNA contamination from tube surface due to air circulated by dry oven fan. Unfiltered and filtered samples were extracted following PPLPP workflow (without optional PCI purification; Figure 2) using 4.4 µg/mL and 55.5 µg/mL in-house glycogen in initial and terminal precipitations, respectively. Initial isopropanol precipitation was conducted at 3,270 x g for 90 min in swinging bucket rotor (Allegra X12R centrifuge with SX4750 rotor; Beckman Coulter Australia). Final 100 µL elution was not subjected to terminal inhibitor purification given the lack of qPCR inhibitors present within *O. mossambicus* tank water (see Figure 4A'). For filter samples, the Longmire's used to preserve each filter replicate half (≈ 1 mL) was recombined in a new 50 mL LoBind® tube, diluted up to 20 mL with DNA-free water, and extracted with PPLPP workflow as described above for unfiltered samples except that, following pellet resuspension, 600 µL Lysis Buffer I (Table 3) was transferred into one 2 mL LoBind® tube that contained both filter halves (see Figure 2 Steps 1c and 9). All samples were assessed for *O. mossambicus* eDNA using Tilapia_v2_16S with subsequent yield extrapolation using *O. mossambicus* gDNA standard curve (Table 4). All assays that produced amplicons with ΔT_m inside 99.7% confidence interval of *O. mossambicus* gDNA standards were considered positive detections without Sanger sequencing confirmation given the sole presence of *O. mossambicus* within sampled tank water.

Results

All unfiltered and filtered *O. mossambicus* tank water samples preserved with Longmire's and subjected to 39 days incubation at room temperature and 50˚C exhibited 100% detection of *O. mossambicus* eDNA (Supplemental Figure 7). One-way ANOVA revealed a significant overall effect on eDNA yield ($F_{4,11} = 5.881$; $P = 0.0088$); however, Dunnett's post-hoc revealed that there were no significant differences relative to baseline ($T = 0$) for unfiltered room temperature and 50˚C samples ($P = 0.0770$ and $P = 0.1304$) or filtered room temperature and 50˚C samples ($P = 0.2338$ and $P = 0.9001$), respectively.



Supplemental Figure S7. PPLPP workflow *in vitro* validation of Longmire's eDNA preservation effectiveness on target species eDNA collected in unfiltered (U) and filtered (F) water samples from *O. mossambicus* tank (see Sources of genomic, synthetic, and environmental DNA) and subjected to six weeks incubation (6wk) at room temperature (RT) and 50˚C (50C). Note that this *in vitro* validation was conducted concurrently with *in situ* validation (see Figure 5 and *In situ* validations). All data presented as total eDNA yield recovered in 100 µL final elution (nanograms ± SEM) based on amplification using Tilapia_v2_16S and extrapolation using *O. mossambicus* gDNA standard curve (Table 4). Baseline *O. mossambicus* eDNA yield (T=0) determined using unfiltered samples extracted using optimal PPLPP workflow immediately following collection (see Longmire's long-term preservation effectiveness). No significant difference in *O. mossambicus* eDNA yield was observed between T=0 and unfiltered or filtered samples incubated at 50˚C for 6 weeks ($P > 0.07$). Bars represent mean ± SEM.

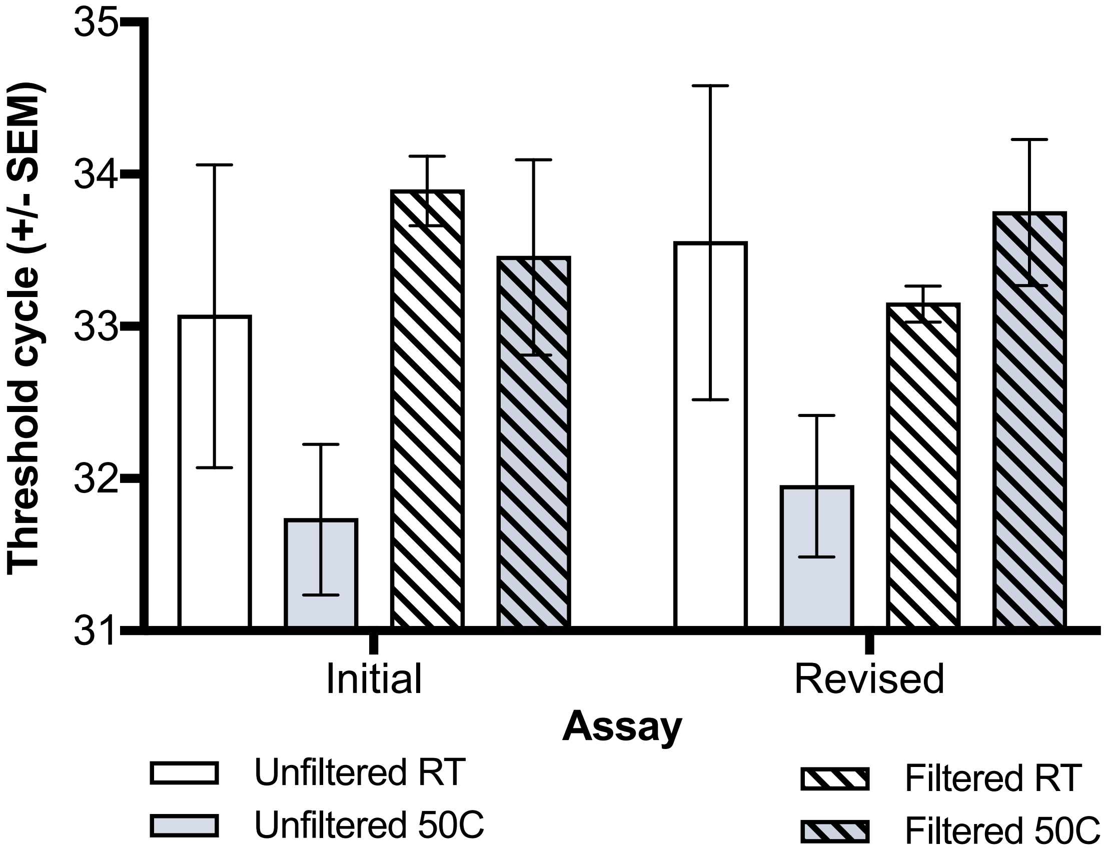
Supplemental Figure S8

Methods

Both initial and revised tilapia assays were run as 10 µL reactions with the same chemistry (PowerUp® qPCR Mastermix; Thermo Fisher Scientific Australia Pty Ltd) and thermal cycling conditions (see Supplemental Information). Fluorescence threshold was set to 0.2 for both assays.

Results

Three-way ANOVA of *O. mossambicus* eDNA detectability using initial versus revised tilapia assays revealed a significant effect of capture method (unfiltered vs filtered; $F_{1,16} = 4.819$; $P = 0.0432$) but no significant effect of assay ($F_{1,16} = 0.02012$; $P = 0.8890$), incubation ($F_{1,16} = 2.384$; $P = 0.1421$), assay X capture ($F_{1,16} = 0.4117$; $P = 0.5302$), assay X incubation ($F_{1,16} = 0.1862$; $P = 0.6719$), capture X incubation ($F_{1,16} = 2.984$; $P = 0.1033$), or assay X capture X incubation ($F_{1,16} = 0.5254$; $P = 0.4790$). Subsequent Sidak post-hoc revealed that there were no significant differences ($P > 0.8$) between initial and revised tilapia assays among all treatments.



Supplemental Figure S8. Comparison of threshold cycle (C_t) values obtained for both unfiltered and filtered *in situ* water samples collected from Ross River (see Sources of genomic, synthetic, and environmental DNA) and screened using both initial and revised (Table 4) tilapia assays (see Supplemental Information). Both initial and revised tilapia assays were run under the same thermal cycling, qPCR chemistry, and analysis conditions (see Supplemental Information). Solid bars represent unfiltered samples while hashed bars represent filtered samples. Three-way ANOVA between initial and revised tilapia assays revealed no significant effect of assays ($P > 0.8$). Bars represent mean ± SEM.

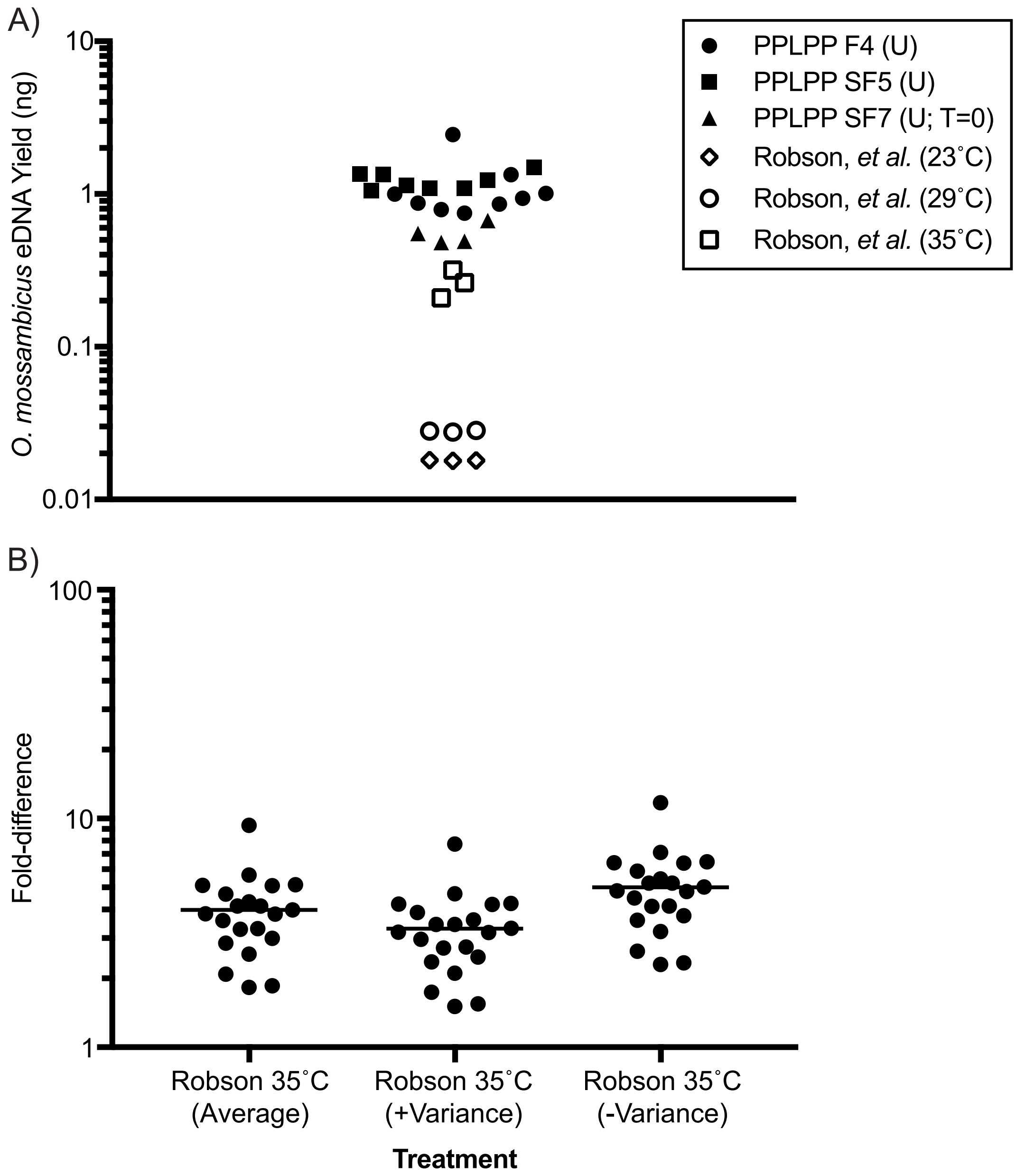
Supplemental Figure S9

Methods

Recovered *O. mossambicus* yields for each unfiltered water sample were compiled across three *in vitro* experiments presented in this study (Figure 4, Supplemental Figures 5 and 7) as well as three *in vitro* temperature treatments (23˚C, 29˚C, and 35˚C) reported in Robson, Noble, Saunders, Robson, Burrows, and Jerry. "Fine-tuning for the tropics: application of eDNA technology for invasive fish detection in tropical freshwater ecosystems". *Molecular ecology resources*. 2016; 16(4): 922-932. Fold-difference in *O. mossambicus* eDNA was calculated between each PPLPP workflow extracted unfiltered water sample and the average, average plus variance, and average minus variance reported for Robson, *et al.* (2016) 35˚C treatment.

Results

Comparison of total *O. mossambicus* eDNA yields across studies revealed that the minimum PPLPP yield (0.48 ng) was greater than the maximum yield of 0.317 ng (average plus variance) reported by Robson, *et al.* (2016) for 35˚C treatment (Supplemental Figure 9A). Subsequent comparison to Robson, *et al.* (2016) 35˚C treatment average (0.263 ng), calculated average plus variance (0.317 ng), and calculated average minus variance (0.209 ng) yields revealed that PPLPP workflow extracted samples contained 3.98 ± 0.5 fold, 3.30 ± 0.4 fold, and 5.01 ± 0.6 fold more *O. mossambicus* eDNA, respectively (Supplemental Figure 9B).



Supplemental Figure S9. Comparison between *O. mossambicus* eDNA yields obtained from unpurified *in vitro* samples presented as part of this study (PPLPP) and reported by Robson, *et al.* (2016) for 23˚C (0.018 ± 0.00007 ng; average ± variance), 29˚C (0.028 ± 0.0003 ng), and 35˚C (0.263 ± 0.054 ng) unpurified *in vitro* treatments. PPLPP samples followed workflow presented here (Figure 2) while Robson, *et al.* (2016) samples were extracted using the benchmark combination of initial EtOH-NaAc precipitation (without co-precipitate) and commercial kit (Table 2). Panel A presents all data points from both studies and demonstrates that the lowest observed yield for PPLPP *in vitro* validations (0.48 ng) is greater than the maximum yield reported by Robson, *et al.* (2016) for 35˚C treatment (0.317 ng). Panel B presents the fold-difference in *O. mossambicus* eDNA recovered from PPLPP *in vitro* validations (3.98-fold, 3.30-fold, and 5.01-fold) compared to Robson, *et al.* (2016) 35˚C treatment average (AVG), average plus variance (+Variance), and average minus variance (-Variance), respectively. Horizontal lines denote mean. Closed circles (●): PPLPP Figure 4 (F4); Closed squares (■): PPLPP Supplemental Figure 5 (SF5); Closed upward triangles (▲): PPLPP Supplemental Figure 7 (SF7) T=0; Open diamonds (◇): Robson, *et al.* (2016) 23˚C treatment; Open circles (○): Robson, *et al.* (2016) 29˚C treatment; Open squares (□): Robson, *et al.* (2016) 35˚C treatment.